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PRINCIPAL INVESTIGATOR: **John Letterio, MD**

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**Case Western Reserve University**
**Cleveland OH 44106-1712**

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**14. ABSTRACT**
It has been suggested that the molecules capable of inducing endogenous antioxidant enzymes through the activation of the Nrf2/ARE pathway may be highly effective in therapeutic and chemopreventive strategies designed to inhibit pathological processes underlying MS lesion formation. Under this proposal we have employed a successful strategy to isolate and characterize one of such compound Triterpenoid from natural plant resources. By applying diversity oriented synthesis we have created several unique triterpenoid structures which will enable careful dissection of the Nrf2-dependent and independent pathways mediating neuroprotection and inflammation and will define a novel class of potent and effective therapeutic and chemopreventive agents for multiple sclerosis. The following progress has been made within the first year of this project:

1) Successful large scale (in gram quantity) isolation of Bryonolic acid from zucchini plant (*Cucurbita pepo* L.) roots.
2) Characterized the activity of isolated Bryonolic acid
3) Applied Diversity Oriented Synthesis to Bryonolic acid to rearrange skeletal structure
4) Analyzed the specificity and activity of skeletally diverse triterpenoids *in vivo* and *in vitro*.

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**INTRODUCTION:**

Although the treatment of multiple sclerosis is complicated by a diverse spectrum of clinical manifestations, recent evidence strongly supports the concept that regulatory factors that skew in association with disease severity may serve as potential targets for new therapeutic interventions. One such regulatory factor is the transcription factor nuclear factor-E2-related factor (Nrf2), a transcription factor that controls expression of genes encoding important antioxidant and stress response proteins through binding to the antioxidant response element (ARE). Nrf2 is strikingly upregulated in the lesions of MS, suggesting the presence of oxidative stress in these lesions. Disruption of Nrf2 gene expression (Nrf2-/- mice) leads to exacerbation of clinical and pathological symptoms of experimental autoimmune encephalomyelitis (EAE), and Nrf2 is reported to be involved in maintaining central nervous system (CNS) myelin. Most importantly, recent evidence supports a concept that molecules capable of inducing endogenous antioxidant enzymes through the activation of the Nrf2/ARE pathway may be highly effective in therapeutic and chemopreventive strategies designed to inhibit pathological processes underlying MS lesion formation.

Triterpenoids are one of the most functionally and structurally diverse classes of secondary metabolites ubiquitous in the plant kingdom. Triterpenoids are cyclized from oxidosqualene to form approximately 200 chemically diverse triterpene skeletons. More than 20 000 triterpenoids have been documented, with new structures continually being identified and studied for their biological activity. In addition to the impressive skeletal diversity of these molecules, they also possess a variety of biological activities including anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, and tonic effects. The central hypothesis of this proposal is that exploration of diverse triterpenoids will enable careful dissection of the Nrf2-dependent and independent pathways mediating immune modulation and neuroprotection and will define a novel class of potent and effective therapeutic and chemopreventive agents for multiple sclerosis.

We became interested in bryonolic acid (BA) (**Figure.-1**) in part because of its unique chemical attributes within the triterpenoid family (namely, the unsaturated B–C ring fusion) and partly due to its interesting pleiotropic profile of biological activity. The activities reported for BA include antiallergic properties, inhibition of homologous passive cutaneous anaphylaxis in rats, delayed hypersensitivity in mice (1,2) antitumor activity, (3) and cytotoxicity toward various tumor cell lines.(3,4) We have shown that BA to be a promising natural anti-inflammatory agent, and mediates it effects by the activation of the transcription factor Nrf2.

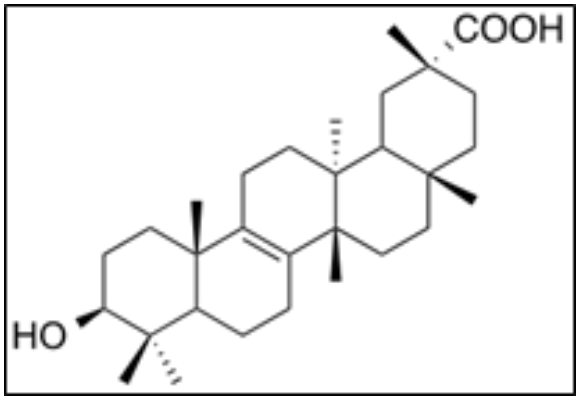



**Fig-1:** Chemical structure of bryonolic acid (BA)

**BODY:**

**1) <u>A large scale isolation of bryonolic acid from Zucchini roots (*Cucurbita pepo* L.)</u>** BA is not commercially available but abundant in many species from the Cucurbita family such as cucumber, watermelon, melon root, culture from radicle. To facilitate further studies of the in vitro and in vivo activity of BA, a protocol for robust isolation of BA is needed. To this end we have developed a reliable and scalable method for isolating gram quantities of BA from the roots of *Cucurbita pepo* L. (*C. pepo* L.), which was chosen based on literature precedence in addition to its commercial unavailability. Although BA has been isolated using alternate methods including callus cell culture, these methods are most appropriate for analytical scale isolation and biosynthetic studies. The key to our approach is a scalable method for obtaining biomass that is rich in BA content. Our initial step in defining our strategy was to determine if BA production was dispersed throughout *C. pepo* L. or limited to specific plant tissues. HPLC traces demonstrate that BA production is confined to the fine hairy root structure of *C. pepo* L. **(Figure 2)**.

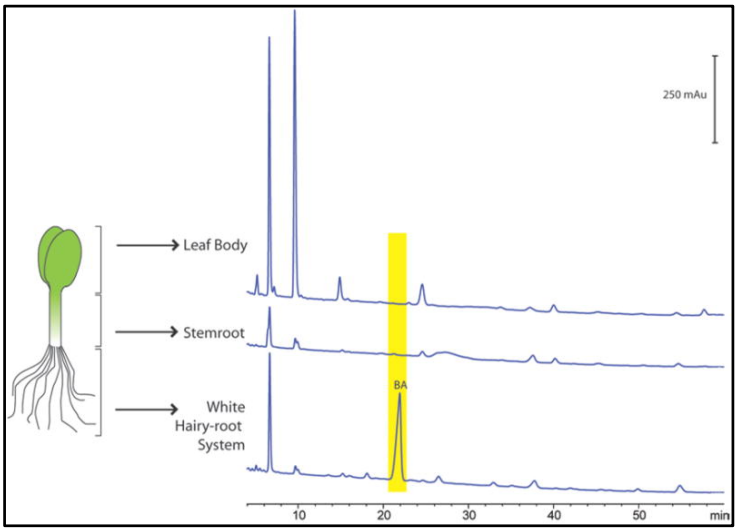



**Fig. 2:** HPLC traces for extracts from the fine hairy root, stemroot, and dicotyledon leaf body of 14 day germinations. Bryonolic acid is detected only in the root portion of *Cucurbita pepo* L.

To address scalability in biomass accumulation we compared two germination methods which are standard in the field: moist blotting paper and peat-based growth media **(Figure 3)**. In both cases, seeds and germinations of *C. pepo* L. were maintained in a medium that retained a moist environment, but did not contribute any level of nutrients to the germinations. For both approaches, roots were isolated every two days and evaluated for BA content by HPLC. For germinations grown in a peat-based media, root isolation was continued for 40 days. The time course and HPLC trace overlay show increasing production of BA from day 2 to day 16, peaking at 1.26 mg $g^{-1}$ dry weight **(Figure 4a, c)**. BA content subsequently decreased from day 16 to day 40, suggesting that BA presence in the roots is most prevalent during the early germination stage.

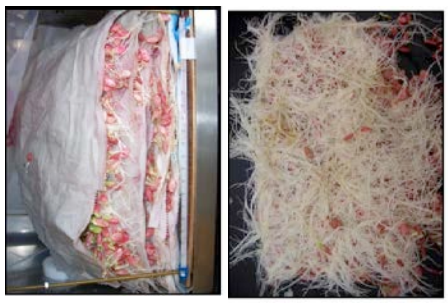


**Fig. 3:** bryonolic acid content in roots from blotting paper is 10-fold greater compared to roots from peat-based media.

In a parallel experiment, germinations were grown between moist blotting paper for 36 days. Germinations were not continued to day 40 as in the peat-based media method due to overgrowth and initial signs of morbidity by day 26. Under these growth conditions, BA content increased from day 2 to day 24 and plateaued thereafter, leveling at approximately 15 mg $g^{-1}$ dry weight **(Figure 4b)**. Presumably, BA content changed little after germinations no longer maintained vitality. The increased BA content can be observed by HPLC peak height on select days between day 2 and day 24 **(Figure 4d)**, and reached a maximum of 16.1 mg $g^{-1}$ dry weight.

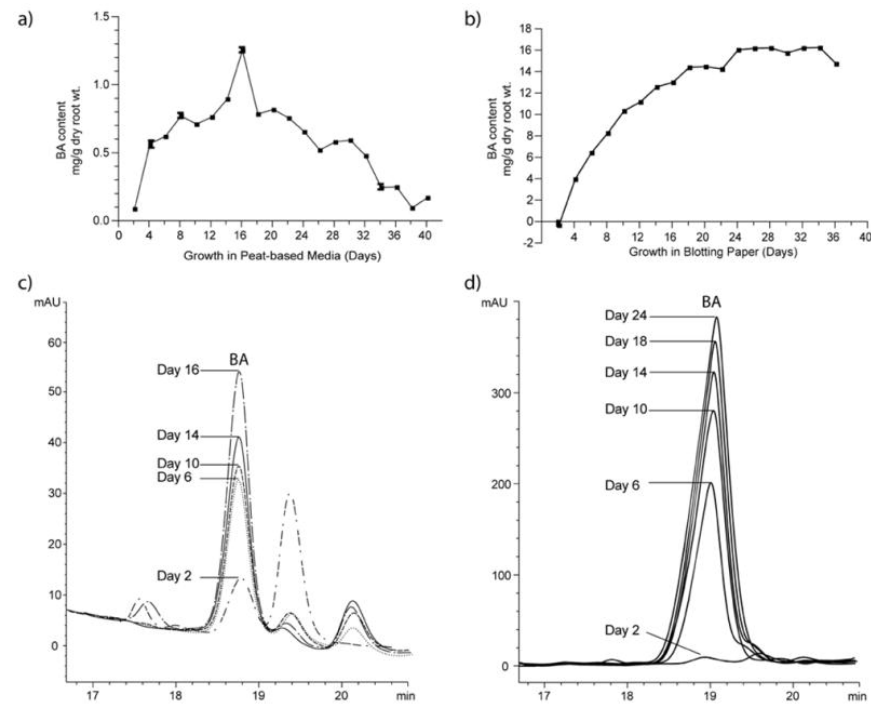



**Fig. 4** Bryonolic acid production in *Cucurbita pepo* L. roots under two growth conditions, peat-based media (a) and moist blotting paper (b). Bryonolic acid content is observed to increase from day 2 to day 16 in peat-based media and from day 2 to day 24 in moist

Taken together, BA content in roots from moist blotting paper is roughly tenfold that detected in roots from peat-based media. Maximum and total BA production per unit mass in roots from blotting paper germinations far outweighed the total amount produced in the peat-based growth media **(Figure 5a)**. On those days resulting in maximum BA content (day 16 for peat-based, day 24 for blotting paper) the magnitude of BA production in germinations grown in blotting paper is apparent in the HPLC trace overlays for these respective days **(Figure 5b)**. In germinations grown in blotting paper, we do not observe a decrease in BA content following achievement of maximum content. This may be due to the capability of retaining 100% of root material when isolated from the blotting paper as opposed to the unavoidable root loss experienced when isolating root material from the peat-based germination media as discussed above.

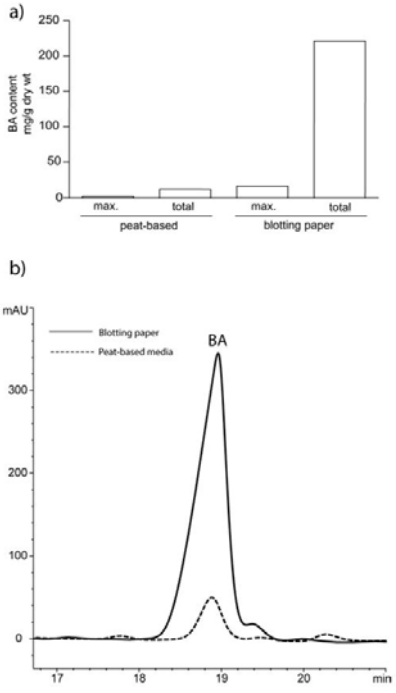



**Fig. 5** Comparison of maximum and total BA production in roots from peat-based media versus roots from moist blotting paper (a). Comparison of HPLC peak area for maximum BA production under both conditions (b).

**2) Analyzing the activity of purified bryonolic acid:** In order to elucidate the anti-inflammatory effects of BA, we used an established in vitro model of LPS-activated RAW 264.7 leukemic mouse macrophage cells (RAW). Upon LPS activation of RAW cells, NO is produced, which spontaneously oxidizes to nitrite. In an initial experiment, LPS-activated RAW cells were treated with BA and nitrite levels were measured from cell culture supernatants.

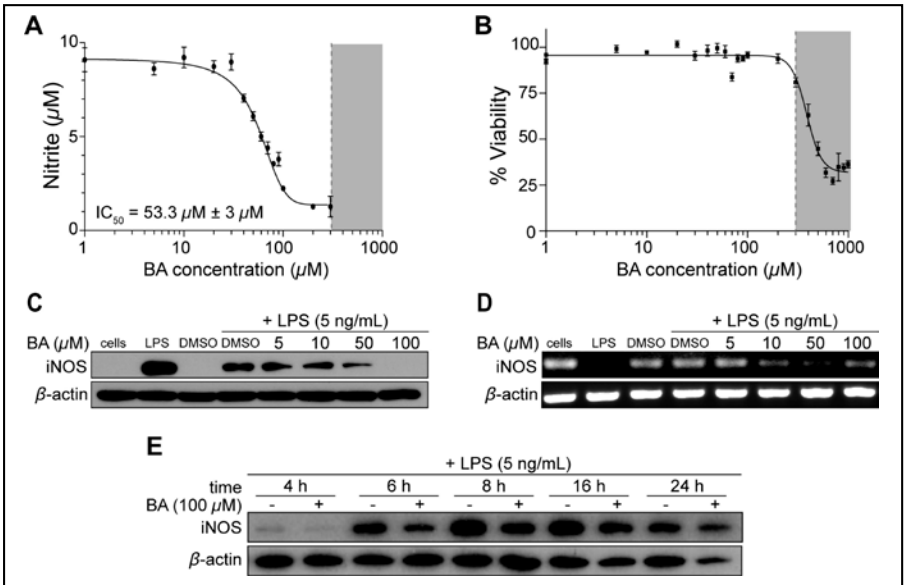



**Fig. 6 :** Bryonolic acid (BA) decreases NO levels and inhibits iNOS expression in RAW 264.7 cells in a dose-dependent and time-dependent manner. RAW 264.7 cells were activated with 5 ng/mL LPS and treated with varying concentrations of BA for 24 h (A–D) or varying time points (E). (A) Nitrite levels were measured via Griess assay in LPS-activated cells treated with BA for 24 h. (B) viability was measured by the MTT assay. (C) iNOS protein levels were quantified through immunoblot analysis in LPS-activated cells treated with varying concentrations of BA (D) iNOS mRNA levels were measured by RT-PCR. (E) iNOS protein levels were quantified through immunoblot analysis in LPS-activated cells treated with 100 μM BA or DMSO control at various time points.

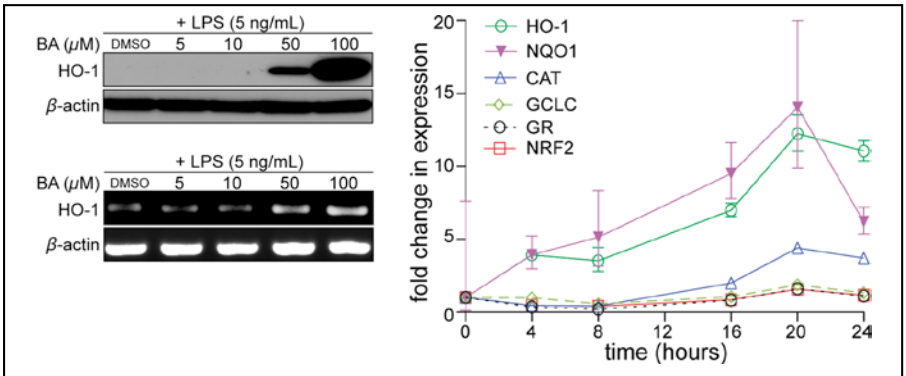



**Fig. 7:** Bryonolic acid (BA) induces HO_1 expression (left panel) and induces Nrf2 with its target genes in in RAW 264.7 cells in a dose-dependent and time-dependent manner.

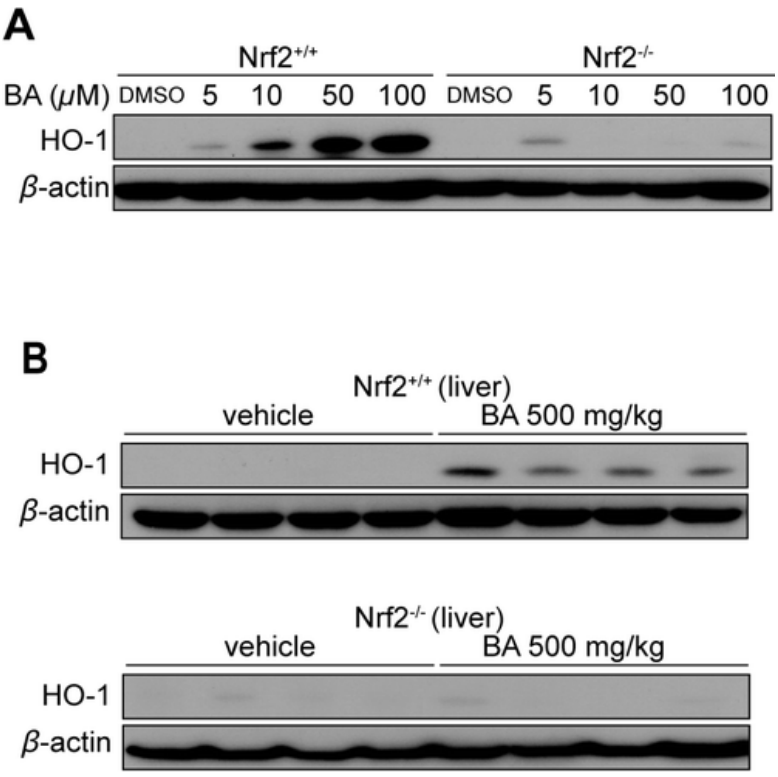



**Fig. 8.** Bryonolic acid (BA) induces HO-1 in primary peritoneal macrophages and liver and is dependent on the Nrf2 pathway. (A) Primary peritoneal macrophages harvested from Nrf2 wild-type or Nrf2 knockout (C57BL/6J background) mice were treated with varying concentrations of BA (**1**) for 48 h. Immunoblot analysis of HO-1 protein levels in BA (**1**)-treated primary macrophages. (B) Immunoblot analysis of HO-1 protein levels in liver tissues of Nrf2 wild-type (upper panel) or Nrf2 knockout (lower panel) mice treated with 500 mg/kg BA or vehicle for 8 h by ip.

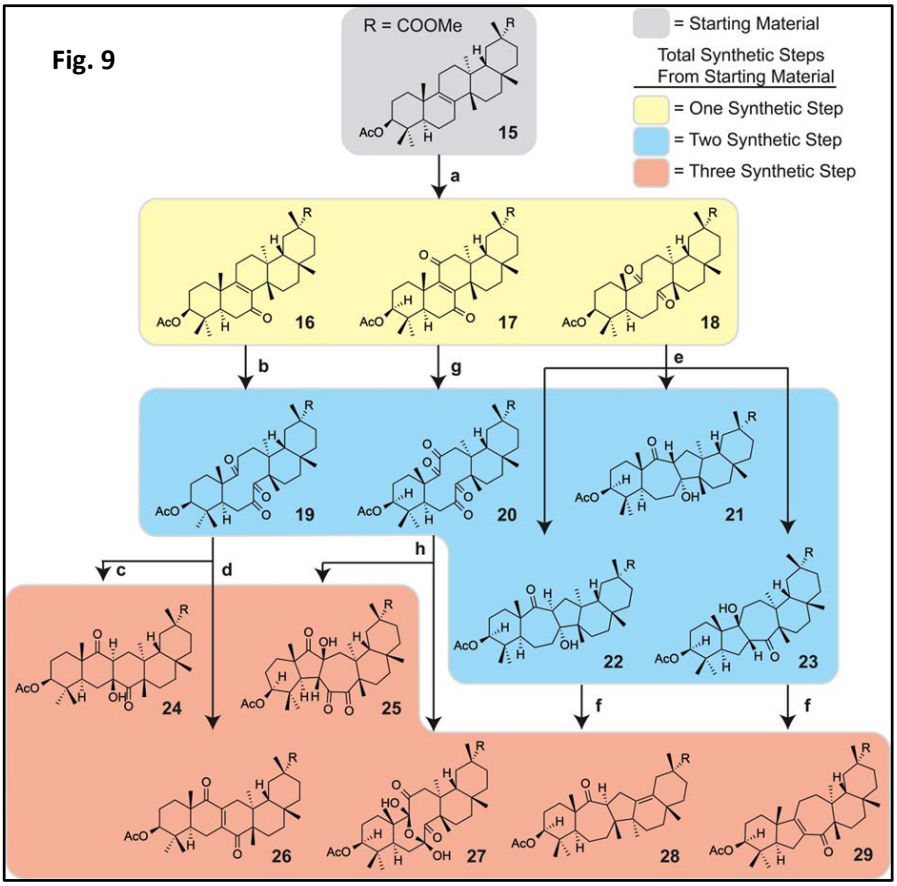
**3) Diversity Oriented Synthesis for efficient skeletal diversification of triterpenoids from purified BA :**

The remodeling of a natural product core framework by means of diversity-oriented synthesis (DOS) is a valuable approach to access diverse/ biologically relevant chemical space and to overcome the limitations of combinatorial-type compounds. Here we adopted a thorough conformational analysis for a general strategy whereby the inherent complexity of our starting material (BA) is used to define the regio- and stereochemical outcomes of reactions in chemical library construction. This is in contrast to the traditional DOS logic employing reaction development and catalysis to drive library diversity.

The approach we adopted in this project deviates from this traditional model in that we set out to identify chemical functionality that can be exploited to rearrange the carbocyclic skeleton of an abundant natural product. The central hypothesis behind our approach was that instead of relying on reaction development and catalysis to

impart stereochemical and regiochemical selectivity, we postulated that the inherent complexity of the natural product-derived substrates can drive stereoselective and regioselective reactions.

**Fig. 9**

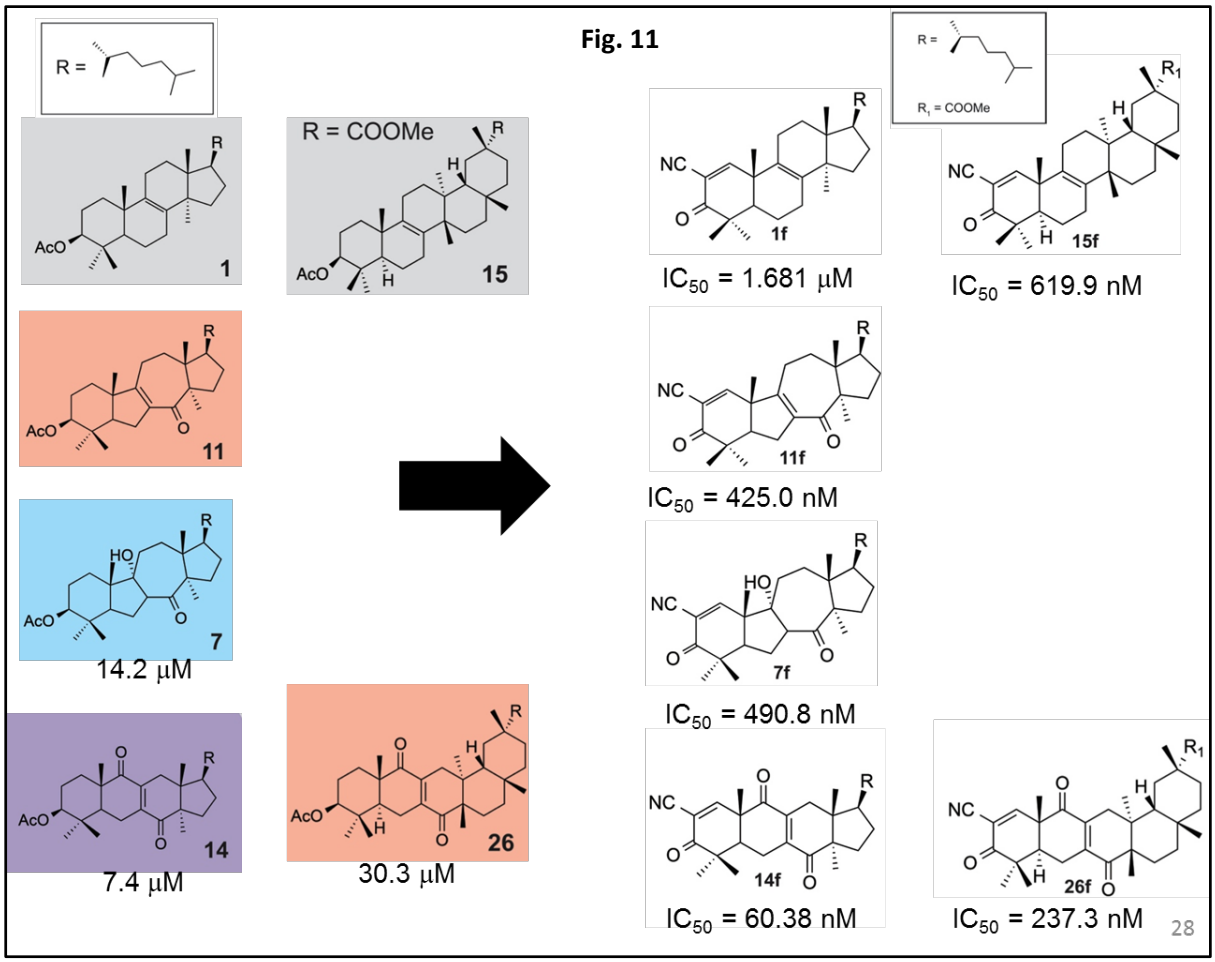



As illustrated above, with a series of steps involved in our DOS chemistry we recovered an array of compounds, few of which are depicted above. We further tested these compounds in our earlier standardized assay (NO suppression in LPS treated RAW 264.7 cells) to test their activity. Invariably we observed a wide spectrum of anti-inflammatory activity in all skeletally diverse molecules with the linear derivatives being one of the most potent. We further optimized the activity of selected most potent compounds from this list. See below:

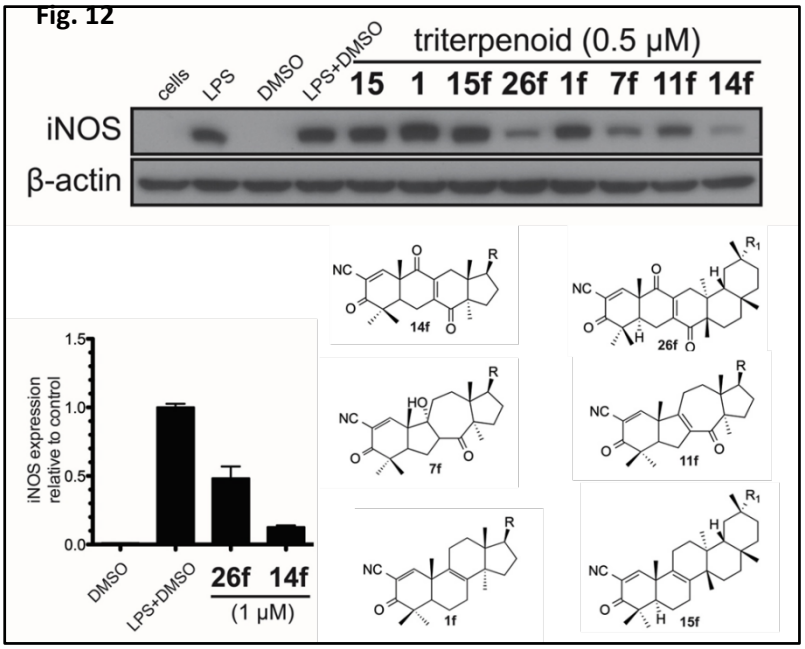
**Fig. 10**

| $IC_{50}$ (μM)[a] | | $IC_{50}$ (μM)[a] | |
|---|---|---|---|
| ***parent molecule*** | | ***parent molecule*** | |
| **1** | inactive | **15** | inactive |
| ***oxidation/oxidative cleavage*** | | ***oxidation/oxidative cleavage*** | |
| **2** | > 100 | **16** | > 100 |
| **4** | > 100 | **17** | > 100 |
| **3** | 53.2 | **19** | > 100 |
| **5** | 11.7 | **18** | 97.0 |
| **6** | 7.9 | **20** | 11.5 |
| ***aldol addition/condensation*** | | ***aldol addition/condensation*** | |
| **11** | > 100 | **22** | > 100 |
| **13** | 25.0 | **25** | > 100 |
| **8** | 17.1 | **28** | > 100 |
| **7** | 14.2 | **23** | 46.3 |
| **9** | 13.0 | **29** | 38.2 |
| **12** | 10.1 | **26** | 30.3 |
| **10** | 8.0 | **21** | 25.6 |
| **14** | 7.4 | **24** | 17.5 |
| | | **27** | 8.3 |

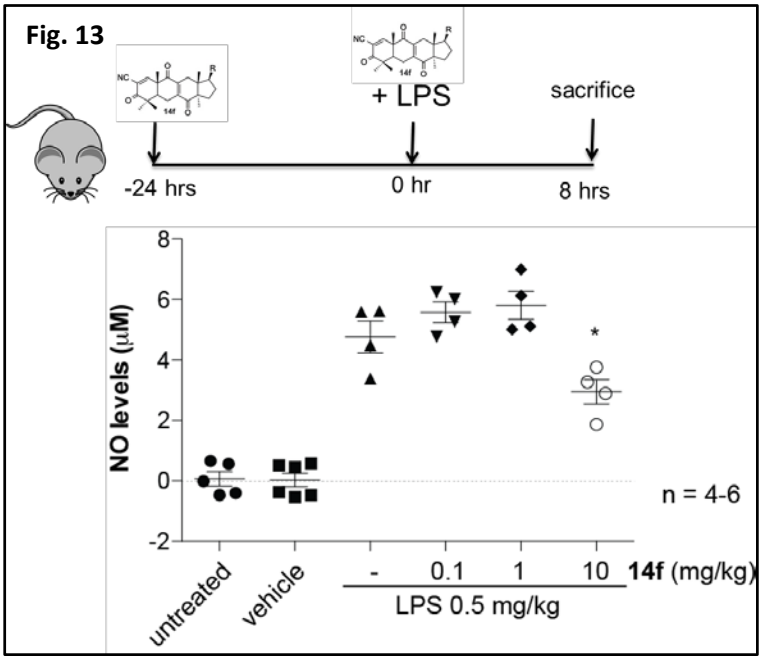
[a]$IC_{50}$ measures the drug concentration required for the inhibition of 50% of nitric oxide after 24 h of incubation.



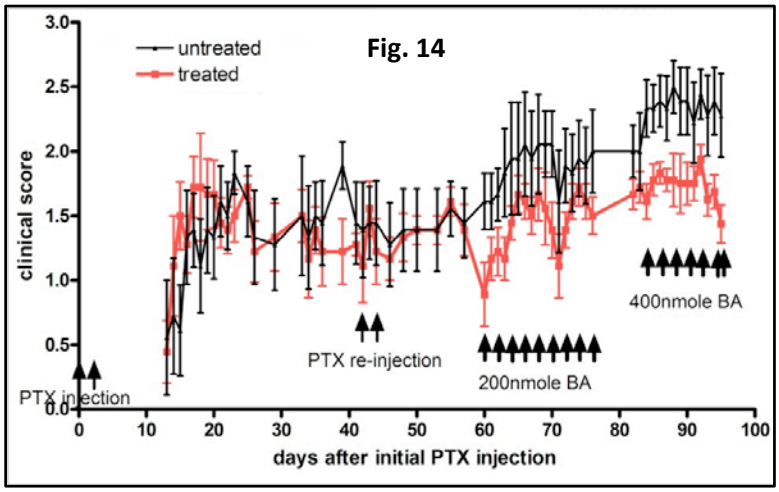

These triterpenoid derivatives significantly inhibited NO production via transcriptional inhibition of iNOS,. For further experiments we will be using most active **26f and 14f** compounds.



We next further tested the in vivo activity of most potent 14f and 26f compound. In brief, mice were injected with LPS treatment along with prior and concurrent treatment of 14f compound. After eight hours blood NO levels were measured by Greece assay. A significant drop in 14f treated mouse group was observed.




In our next series of experiments we will be testing the bio efficacy of these compounds in murine model of multiple sclerosis EAE (experimental autoimmune encephalomyelitis). To move further and test our set of newly derived compounds in this model we first wanted to test the efficacy of the parent compound (BA) itself in the treatment of EAE. EAE was induced in 17 mice by MOG-35-55 immunization along with two pertussis toxin injections. Eight mice were treated with depicted doses of BA and nine remained vehicle treated. During a course of 3 months treatment we observed a slight improvement in disease mean clinical scores. These results are encouraging as we know that BA is not as active as its newly synthesized derivatives. We are in process of synthesizing more amount of active derivatives and will be testing them in similar experimental settings.



## KEY RESEARCH ACCOMPLISHMENTS:

Within the first year of this project period we have accomplished the following:

1) Developing a method to isolate large quantities of BA from a plant source
2) Establishing the biological activity of purified BA both in vivo and in vitro
3) Development of more potent active compound by applying DOS chemistry on parent BA
4) Establishing the Nrf2 mediated mode of action of the selected compounds

## REPORTABLE OUTCOMES:

A key strength of this proposal is the interdisciplinary research team of the Tochtrop and Letterio laboratories. Our two labs are independently interested in triterpenoids: the Tochtrop lab from the point of view of the chemistry, biochemistry, and biodiversity and the Letterio lab from the point of view of immune modulation, preclinical disease models, and disease therapy and prevention. This collaboration is cemented when two graduate students (Tonibelle Gatbonton-Schwager & Emily Barker) committed to joint projects. With the postdoctoral leadership provided for both the chemical (Yong Han) and biological (Tej Pareek) aspects of the proposal, we have a very strong research team to complete the outlined studies. The most part of the work presented here will be integral part of the PhD thesis of Tonibelle Gatbonton-Schwager & Emily Barker.

## CONCLUSION:

- Diversification of the triterpenoid skeletal structure through DOS resulted in molecules with a wide spectrum of anti-inflammatory and antioxidant activity.
- Core skeleton structure of triterpenoid dictates activity.
- Skeletal rearrangement of the carbocyclic triterpenoid in combination with optimization of the A-ring resulted in the identification of effective and structurally diverse Nrf2 activators.
- The linear compounds are more active than their counter parts. We have identified two most potent compounds 14f and 26f, which we will use for our further studies.

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